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基质对中国特有濒危蕨类中国蕨配子体形态发育和繁殖的影响

王卫清[1,2]，成 晓[1*]，焦 瑜[1]，陈贵菊[3]
（1 中国科学院昆明植物研究所，云南 昆明 650204；2 中国科学院研究生院，北京 100049；
3 济宁市农业科学研究院，山东 济宁 272031）

摘要：采用4种培养基质对中国蕨（*Sinopteris grevilleoides*）孢子进行培养，结果显示：不同基质上中国蕨配子体和幼孢子体生长发育存在差异，尤其是心形原叶体的形态差别明显；腐叶土基质上的心形原叶体上未见颈卵器的发生，因而其有性繁殖过程不能完成；原生境土和腐叶土1∶1混合土基质最适合中国蕨配子体生长发育及有性繁殖；改良Knop′s琼脂培养基上要保证幼孢子体正常生长需追加培养液。

关键词：中国蕨；配子体；形态发育；繁殖；培养基质
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Substratum Affects the Gametophyte Development and Reproduction of *Sinopteris grevilleoides* (Sinopteridaceae), an Endangered Fern Endemic to China

WANG Wei-Qing[1,2], CHENG Xiao[1*], JIAO Yu[1], CHEN Gui-Ju[3]
(1 *Botanical Garden, Kunming Institute of Botany, Chinese Academy of Sciences*, Kunming 650204, China;
2 *Graduate University of Chinese Academy of Sciences*, Beijing 100049, China;
3 *Jining Institute of Agricultural Sciences*, Jining 272031, China)

Abstract: *Sinopteris grevilleoides* is an endangered fern endemic to the region of Yunnan and Sichuan in southwest China. In this study, we investigated the effects of four different culture substrata on the development of gametophyte and reproduction of this fern. Cultured on different culture substrata, the fern showed different morphology of gametophyte. In humus soil, cordate prothalli did not bear archegonia, and thus the process of sexual reproduction of *S. grevilleoides* could not be finished. The substratum composed of humus soil and original soil in ratio of 1∶1 was the most suitable substratum for gametophyte development and sexual reproduction of this species. Otherwise, infant sporophytes needed extra nutrient solution to sustain its growth on improved Knop′s agar medium.

Key words: *Sinopteris grevilleoides*; Gametophyte; Morphological development; Reproduction; Culture substratum

Sinopteris grevilleoides (Christ) C. Chr. et Ching (Fig. 1), a rare and endangered species endemic to China, has a scattered distribution only in Sichuan and Yunnan in southwest China (Ching and Shing, 1990). Declining rapidly in population size and on the verge of extinction, it has been listed as a plant of second-class protection in *The List of Chinese Urgently Protected*

* Author for correspondence; E-mail: xcheng@mail.kib.ac.cn; Tel: + 86-871-5223903
Received date: 2009-07-28, Accepted date: 2009-11-23
作者简介：王卫清（1982－）女，在读硕士研究生，主要从事蕨类植物保育生物学研究。

Wild Plants (Yu, 1999). Previous studies of this species mostly focused on its system position (Guo *et al.*, 1992; Zhang *et al.*, 2007), while little work has been done on its gametophyte development and reproduction strategy.

The effects of culture substratum on gametophyte morphological development and reproduction of *Sinopteris grevilleoides* were investigated here. Gametophyte and infant sporophyte development and the reproduction characteristics of the species on four different culture substrata were reported. The information obtained will provide a basis for its artificial propagation and conservation.

1 Materials and Methods

1.1 Spore collection

Spores were obtained from fertile fronds of sporophytes. Pinnae were left to dry at room temperature in paper envelopes to facilitate the opening of the sporangia and expulsion of the spores. Spores were separated from fragments of fronds and sporangia, then stored in refrigerator at about 4℃. Voucher specimens are deposited in the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN).

1.2 Soil culture

For Culture 1－3, spores were sown in three different soil substrata: humus soil, mixture soil composed of humus soil and original soil in ratio of 1∶1 and original soil, which were marked as Culture 1, Culture 2 and Culture 3 respectively. The humus soil was collected under the mixed coniferous broad leaved forest in Yunnan Province. The original soil was collected from the habitat of *Sinopteris grevilleoides* - Dayao, Yunnan. The results of chemical property analysis of the two kinds of soil are presented in Table 1. The pH of the mixture soil was 6.0－6.5. The soil substrata were sterilized by sautéing in the Chinese way for forty minutes at the temperature of 140－160℃, and then sub-packaged in 12 cm diameter petri dishes after cooled. The depth of soil in the petri dishes was about 0.5 cm. The surface of the substratum was kept smooth and substantial, and then the soil was soaked by cool boiled water. Finally, the spores were rinsed with sterilized water for four times, and then sown evenly at an average density of 200－300 spores per cm^2.

Table 1 The results of chemical property analysis of the soil (mean±SE)

Testing items	Original soil	Humus soil
pH	6.58±0.01	4.23±0.03
Organic matter (%)	3.736±0.03	36.08±0.06
Total N (%)	0.199±0.00	0.93±0.01
Total P (%)	0.071±0.00	0.14±0.00
Total K (mg/kg)	1.747±0.00	0.23±0.00
Available N (mg/kg)	124.76±4.93	231.87±3.01
Available P (mg/kg)	7.01±0.21	56.77±2.36
Available K (mg/kg)	157.66±2.89	404.73±2.97
Potent Ca (mg/kg)	4462.65±0.01	468.96±4.18
Available Mg (mg/kg)	264.30±0.01	175.89±0.54
Available Zn (mg/kg)	1.77±0.01	16.09±0.20
Available Fe (mg/kg)	9.53±0.01	18.71±0.22
Available (mg/kg) B	0.81±0.03	1.10±0.00
Available S (mg/kg)	33.80±1.54	13.85±0.12

(Mean and SE numbers in the above table were calculated with the results of three parallel tests)

1.3 Improved Knop's agar medium culture

For Culture 4, the spores were sterilized with 4% sodium hypochlorite for 5 min then rinsed with sterilized water four times; between rinses spores were centrifuged at 3500 r/min for five times (AD-72 centrifuge). After that, the spores were sown evenly at an average density of 200－300 spores per cm^2 in 6 cm diameter petri dishes. The medium was improved Knop's agar medium (Liu *et al.*, 1991) and the pH of this medium type was 6.8.

Every culture was set fifteen repetitions. All cultures were kept in the dark at 25℃ for 24 h then transferred to the lab with artificial light (pink fluorescent illumination) at 1000－1500 lx under a diurnal cycle of 12/12 h. The temperature was 22－28℃ during illumination and 14－18℃ in dark. Through the entire process, soil cultures were moistened with cool boiled water to prevent desiccation, and to help the opening of antheridia and the movement of antherozoid in the later stage. On the original soil (Culture 3), prothalli, which stopped growing after bearing antheridia and archegonia, were transplanted from three petri dishes to one petri dish, and then were sprayed with water twice a day.

Morphological development was observed under the optical microscopes (OLYMPUS BX51) and anatomical lens (OLYMPUS SZ61). For observations, gametophytes and infant sporophytes were removed from culture and mounted in water. Morphological characteristics of fresh gametophytes were recorded with optical CANNON POWERSHOT A640 camera.

2 Results

The gametophyte morphological development of *Sinopteris grevilleoides* on different culture substrata varied and the details were showed in Table 2. The spores on the mixture soil composed of humus soil and original soil in ratio of 1∶1 (Culture 2) began to germinate at 8—12 days after sown, similar to the spores on the Knop′s agar medium (Culture 4), 8—15 days earlier than that on the humus soil (Culture 1), and 1—9 days earlier than that on the original soil (Culture 3). The rates of spore germination in Culture 2 and Culture 4 were over 67% (Table 2).

Filaments all had 3—7 cells and rarely branched on the three kinds of soil media, while bifurcation of filaments on the Knop′s agar medium (Fig. 9) was apparent-the rate of branched filament reached up to 25%—30% (Table 2).

The morphology of cordate prothalli on different culture substrata differed significantly from each other. The cordate prothalli elongated and the margins of the wings were slightly curved on the humus soil (Culture 1) (Fig. 2). The cordate prothalli grew wider with the smooth wing margins on the mixture soil (Culture 2) (Fig. 3). The shape of cordate prothalli on the original soil (Culture 3) was similar to that on the mixture soil but a little longer (Fig. 4). On the Knop′s agar medium, the prothalli are butterfly-shaped with flexuous wing margins (Fig. 5).

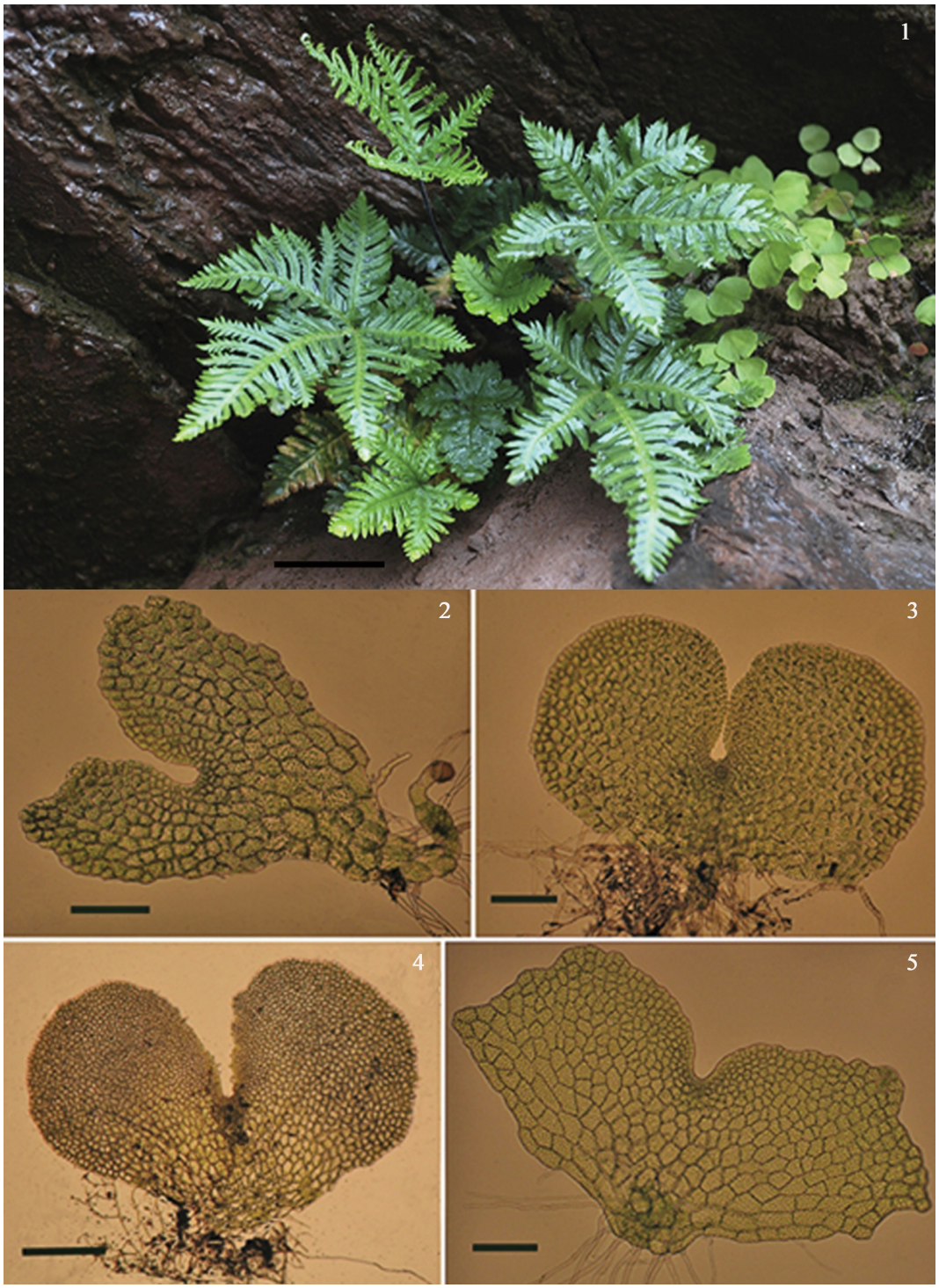
The prothalli were bisexual although the antheridium and archegonium occurred on each prothallus asynchronously. The prothalli on the humus soil no longer developed archegonia after they had given birth to antheridia and the prothalli grew continuously into large irregular green lamina (Fig. 6) by keeping the soil substratum′s moisture at more than 60%.

On the original soil (Culture 3), most prothalli stopped growing after bearing antheridia and archegonia; the formation rate of young sporophytes was only 20%, even though the culture moisture was kept at more than 90%; when all the prothalli in three petri dishes were transplanted to one petri dish, the rate was improved from 20% to 60%. The prothalli had born antheridia before they grew into cordate on the Knop′s agar medium (Fig. 8). The prothalli on the mixture soil medium usually produced more antheridia and archegonia than those on the agar medium.

Table 2 Gametophyte morphological development of *S. grevilleoides* on four culture substrata

Gametophyte morphogenesis	Culture 1 (humus soil)	Culture 2 (humus soil: original soil = 1: 1)	Culture 3 (original soil)	Culture 4 (Knop′s agar)
Spore germination (d)	20—23	8—12	13—15	7—11
Rate of spore germination (%)	10—15	67—73	18—20	68—75
Number of filament cells (unit)	3—7	3—7	3—7	4—8
Ratio of branched filament (%)	0	0	0	25—30
Second dimensional growth (d)	29	14	20	12
Cordate prothallus (d)	37	29	32	25
Ratio of the length and width of the prothallus	1.4—1.6	0.6—0.8	1.1—1.3	≤0.5
Antheridia appearance (d)	46	30	45	23
Antheridia number (u/p)	14—16	20—57	16—20	17—24
Archegonia appearance (d)	Non	50	74	46
Archegonia number (u/p)	Non	11—20	7—13	6—14
Infant sporophyte appearance (d)	Non	93	122	114
Rate of prothallus developing into sporophyte (%)	0	70	20	60

d=day (number of days from seeding spores to firstly observing every stage of gametophyte morphological development), u=unit, p=prothallus. (All values in the above table were mean values of results of at least ten cyclical tests)

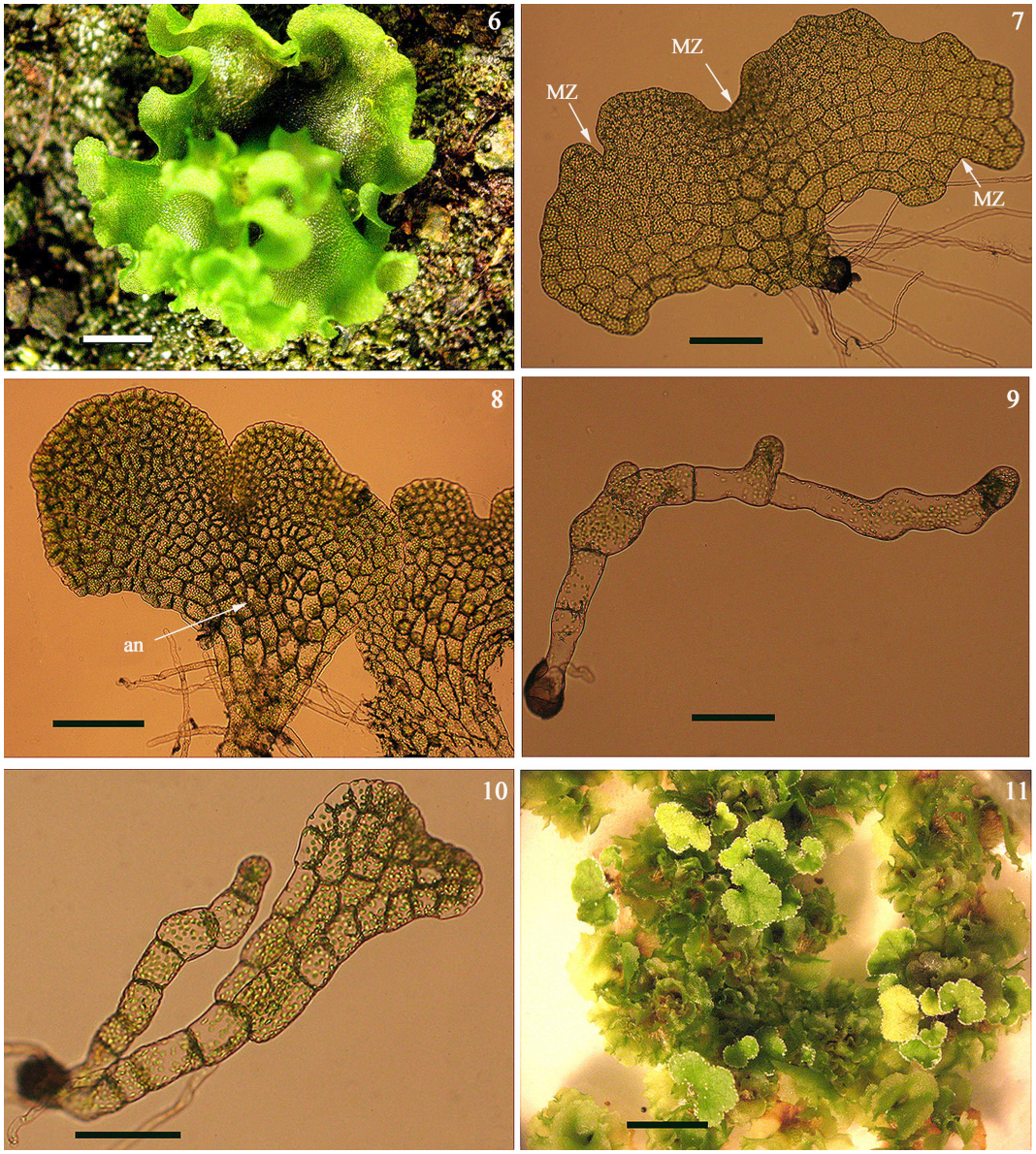


Figs. 1—5 Cordate prothalli

1. Habitat of *S. grevilleoides* (Dayao, Yunnan, China, alt. 1 350 m, Aug. 4, 2008); 2. (Culture 1, 37 d); 3. (Culture 2, 25 d); 4. (Culture 3, 32 d); 5. (Culture 4, 29 d). d=day (days from seeding spores to firstly observing cordate prothalli). (1: bar=3 cm; 1—5: bar=150 μm)

On the Knop′s agar medium (Culture 4), some spores developed into two filaments and the filaments both grew into prothalli (Fig. 10); the case of one prothallus with more than one meristematic zone frequently occured (Fig. 9); infant sporophytes stopped growing after the first primary

leaf or the second one came out (Fig. 11) and their constant growth needed extra nutrient solution; the rate of prothalli developing into saprophytes reached up to 70%.



Figs. 6—11　Gametophyte morphology of *S. grevilleoides*

6. Large irregular green lamella (Culture 1, 48 d, bar=160 μm); 7. Prothallus with several meristematic zones (Culture 4, 38 d, bar=120 μm); 8. Non-heart-shaped prothallus with antheridias (Culture 2, 20 d, bar=120 μm); 9. Bifurcate filament (Culture 4, 25 d, bar=50 μm); 10. Filament and lamellar structure developing from one spore (Culture 4, 30 d, bar=70 μm); 11. Infant sporophytes (Culture 4, 155 d, bar=200 μm).

d=day (days from seeding spores to firstly observing the morphology shown); an=antheridium; MZ=meristematic zone

3　Discussion

Our observation showed that the mature cordate prothalli on the humus soil didn't develop archegonia, and, thus, the normal process of sexual reproduction of *S. grevilleoides* couldn't finished. Obviously, the humus soil was not suitable for the spore propagation of this fern. That the pH of the humus soil was too low was

probably the reason. On the mixture soil composed of humus soil and original soil (1 ∶ 1) (Culture 2), the spores germinated and developed earlier than that on the humus and original soil, and the rates of spore germination and prothallus developing into sporophyte were both the highest among the four cultures. The mixture soil provided the fern not only with suitable pH 6.0—6.5, but also with abundant organic matter and available mineral elements such as N, P, and K. Thus the mixture soil is most favorable for the spore propagation of this species among the three examined soil substrata.

Gametophytes of *S. grevilleoides* all formed nearly symmetrical, cordate prothalli at a young stage though the shapes of prothalli on each medium were not all the same. It was reported that the growing density influenced the shape of prothallus (Cao *et al.*, 2003). It was also showed that the contents of N. P. K, and Ca in the soil affect the early development of the fern reproduction by spores (Suzuki *et al.*, 2005). In our study, though the spores all were sown evenly at an average density of 200—300 spores per cm^2 for each culture, the rate of spore germination on four culture substrata was different, which made the density of cordate prothalli on each culture substratum differ. It was unclear that which factor, different density of cordate prothalli or different physical and chemical nature of each substratum, resulted in different shapes of cordate prothalli.

The most common sequence of sexual development in homosporous ferns involves the formation of antheridia followed by archegonia (Atkinson and Stokey, 1964). However, Prada *et al.* (2008) reported that in the case of *Pteris incompleta* progression of sex expression varied depending on the culture medium, namely, female gametophytes developed earlier than male ones on soil cultures. In this research, the sequence of sexual expression appeared to be independent of substrate type. The male gametophytes developed initially and the female ones later, which brought into correspondence with most taxa (Haufler and Ranker, 1985).

On the original soil, the rate of prothallus developing into sporophyte improved greatly after transplanting; accordingly, it was more than likely the previous low formation rate of sporophyte was caused by the low density of cordate prothalli on the substratum, and the low density of prothalli was resulted from the low rate of spore germination on the original soil.

Stokey (1951) reported that unfavorable conditions promoted filamentous growth. In this study, on the Knop′s agar medium, the spores of *S. grevilleoides* germinating later tended to grow long filaments or one spore developed into two filaments and some filaments always branched. The possible reason was the spores germinating later lacked adequate nutrients and space. According to Table 2, the antheridia and archegonia on the Knop′s agar medium (Culture 4) appeared earlier than those on the mixture soil (Culture 2), however, infant sporophytes in Culture 4 appeared later than those in Culture 2. This indicated that the higher density of antheridia and archegonia could promote the emergence of infant sporophytes. Unlike the investigation of Chen *et al.* (2008) —the prothalli on the improved Knop′s agar medium rarely produced infant sporophytes, about 60% of prothalli on the agar medium in this study produced infant sporophytes. This may be caused by the differences between species. The infant sporophytes stopped growing unless the nutrition was sufficient in Culture 4.

Prothalli of most fern taxa are capable of regenerating new prothalli from old ones (Atkinson and Stokey, 1964; Nayar and Kaur, 1971). In this study, the large irregular green lamina on the humus soil (Fig. 6) and the prothallus with several meristematic zones on the Knop′s agar medium

(Fig. 7) suggested that *S. grevilleoides* may regenerate new prothalli from old ones, but additional observation was needed to prove it.

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